

# Angiotensin II induces apoptosis in rat glomerular epithelial cells

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NORA GIBBONS,<sup>1</sup> BALAKUNTALAM S. KASINATH,<sup>3</sup> AND PRAVIN C. SINGHAL<sup>1</sup>

<sup>1</sup>Department of Medicine, Long Island Jewish Medical Center, The Long Island Campus for the Albert Einstein College of Medicine, New Hyde Park, New York 11040; <sup>2</sup>Department of Medicine, Renmin Hospital, Medical College of Wuhan University, Wuhan, Hubei 430060, China; and <sup>3</sup>Department of Medicine, University of Texas Health Science Center, San Antonio, Texas 78284

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**Ding, Guohua, Krishna Reddy, Aditi A. Kapasi, Nicholas Franki, Nora Gibbons, Balakuntalam S. Kasinath, and Pravin C. Singhal.** Angiotensin II induces apoptosis in rat glomerular epithelial cells. *Am J Physiol Renal Physiol* 283: F173–F180, 2002. First published January 8, 2002; 10.1152/ajprenal.00240.2001.—ANG II has been shown to modulate kidney cell growth and contribute to the pathobiology of glomerulosclerosis. Glomerular visceral epithelial cell (GEC) injury or loss is considered to play a pivotal role in the initiation and progression of glomerulosclerosis. In the present study, we investigated the effect of ANG II on GEC apoptosis. Rat GECs were incubated with increasing doses of ANG II for variable time periods. Apoptosis was evaluated by cell nucleus staining and DNA fragmentation assay. ANG II induced GEC apoptosis in a dose- and time-dependent manner. The proapoptotic effect was attenuated by the ANG II receptor type 1 antagonist losartan or the ANG II receptor type 2 antagonist PD-123319 and was completely blocked by incubation with the combined antagonists. Moreover, ANG II stimulated transforming growth factor (TGF)- $\beta$ 1 production as measured by ELISA. GECs exposed to TGF- $\beta$ 1 demonstrated a dose- and time-dependent increase in apoptosis. ANG II-induced apoptosis was significantly inhibited by addition of anti-TGF- $\beta$ 1 antibody. ANG II also upregulated the expression of Fas, FasL, and Bax and downregulated the expression of Bcl-2 in GECs. These studies suggest that ANG II induces GEC apoptosis by a mechanism involving TGF- $\beta$ 1 expression that may, importantly, contribute to the pathogenesis of glomerulosclerosis.

angiotensin II; glomerular epithelial cells; transforming growth factor- $\beta$ ; apoptosis; glomerulosclerosis

GLOMERULOSCLEROSIS IS A COMMON pathological process in the progression of renal injury and is characterized by glomerular cell loss and accumulation of extracellular matrix (ECM) proteins (11, 19, 28). Numerous clinical and experimental studies have implicated the renin-angiotensin system (RAS) in the pathogenesis of this pattern of glomerular abnormalities (23, 43, 47). ANG II, the effector molecule of RAS, is known to exert various actions in diverse tissues and cells. ANG II contributes to glomerular pathobiology through not

only its hemodynamic effects but also its nonhemodynamic effects (or direct effects) on glomerular growth and sclerosis (2, 10, 43). ANG II stimulates ECM protein synthesis in cultured mesangial cells by induction of transforming growth factor (TGF)- $\beta$  expression (15). In vivo administration of ANG II to animals significantly increases the production of ECM proteins and expression of TGF- $\beta$  in the glomerulus (15). Inhibition of RAS with angiotensin-converting enzyme inhibitors and ANG II receptor antagonists attenuates the progression of glomerulosclerosis (18, 40). ANG II blockade also decreases the expression of TGF- $\beta$ . The interaction between ANG II and TGF- $\beta$  has been considered to play an important role in human and experimental models of glomerulosclerosis (5).

Recent investigations suggest that an altered balance between cell survival and cell death may result in a loss of glomerular cells, which underlies the development of glomerulosclerosis (13, 33, 35). Apoptosis in glomerular cells has been demonstrated in human diseases (39) and experimental models of the remnant kidney (13, 39), diabetes (48), and hypertensive nephrosclerosis (46), where the increased activity of ANG II may be implicated. However, there are no data on whether ANG II can induce apoptosis in glomerular cells in vivo or in vitro. The mechanism of glomerular cell apoptosis induction remains unknown. In addition to its effect on cell proliferation and growth, ANG II has been shown to induce apoptosis in a variety of human and animal cells in culture, such as vascular smooth muscle cells (44), myocytes (16), fibroblasts (45), endothelial cells (7), and alveolar epithelial cells (42). ANG II-induced apoptosis is believed to be mediated by the ANG II type 2 (AT<sub>2</sub>) receptor, but there are also reports of the ANG II type 1 (AT<sub>1</sub>) receptor mediating apoptosis (4, 45).

Glomerular visceral epithelial cells (GECs), or podocytes, are one of the major cell types in the glomerulus. Increasing evidence suggests that GEC injury or loss plays a pivotal role in the initiation and progression of

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glomerulosclerosis (17, 20, 29, 32). GECs are situated on the glomerular basement membrane and presumably are subjected to the direct effects of intrarenal ANG II activation under pathological conditions. GECs have been documented to express both AT<sub>1</sub> and AT<sub>2</sub> receptors (36). However, a few studies have been carried out to evaluate the effects of ANG II on cellular functions and structure in GECs (12, 37), but the question of whether ANG II affects survival of GECs still remains unknown. Therefore, the present investigation was designed to study the effect of ANG II on apoptosis in cultured GECs and to evaluate the effects of ANG II receptor antagonists and TGF- $\beta$ 1 on cell apoptosis in response to ANG II.

## METHODS

**GEC culture.** GECs were cultured from isolated glomeruli of Sprague-Dawley rats (Harlan, Indianapolis, IN) as previously described (9, 31, 34). Briefly, glomeruli were isolated by differential sieving of minced cortices, collagenase digested, and plated. Early cellular outgrowths at 5–7 days were selectively removed by a cylinder cloning technique. Cells were replated, and plates growing pure colonies were then expanded. GECs were identified by currently recognized criteria, including their characteristic polygonal appearance as seen on phase-contrast microscopy, sensitivity to puromycin aminonucleoside, positive stain for podocalyxin, heparan sulfate proteoglycan core protein, and vimentin, and negative stain for myosin and factor VIII. The cells were maintained in culture with media containing 50% DMEM, 50% Ham's F-12 (GIBCO BRL, Grand Island, NY), 5% Nu-serum (Collaborative Biomedical Products, Bedford, MA), 5  $\mu$ g/ml insulin, 5  $\mu$ g/ml transferrin, and 5  $\mu$ g/ml selenium (ITS; Collaborative Biomedical Products) at 37°C in 5% CO<sub>2</sub>. GECs were used for experiments between passages 8 and 12.

**Experimental treatments of GECs.** Equal numbers of GECs were plated and grown to subconfluence. Cells were washed twice with PBS and then incubated in media (+1% FCS) containing buffer (control) or variable concentrations of ANG II (Sigma, St. Louis, MO), ANG III, ANG IV (3–8), ANG-(1–7) (Bachem Bioscience, King of Prussia, PA), or TGF- $\beta$ 1 (R&D Systems, Minneapolis, MN) for the indicated times. In separate experiments, GECs were treated with ANG II in the presence of losartan (Merck, Rahway, NJ), PD-123319 (a selective AT<sub>2</sub> receptor antagonist; Parke-Davis Pharmaceutical, Ann Arbor, MI), or losartan+PD-123319 or anti-TGF- $\beta$ 1 antibody (R&D Systems) for 1, 18, or 24 h.

**Detection of apoptosis by cell nucleus staining.** Morphological evaluation of GEC apoptosis was carried out by staining the cell nucleus with H-33342 (Molecular Probes, Portland, OR) and propidium iodide (Sigma). H-33342 stains the nuclei of live cells and identifies apoptotic cells by increased fluorescence, whereas propidium iodide contains the necrotic cells (pink). Double staining by these two dyes provides the percentage of live, apoptotic, and necrotic cells under control and experimental conditions (38). Briefly, at the end of the incubation period as described in *Experimental treatments of GECs*, cells were washed and stained with H-33342 (1  $\mu$ g/ml) for 7 min at 37°C. Then, cells (without a wash) were placed on ice with the addition of propidium iodide (final concentration of 1  $\mu$ g/ml). Cells were incubated with both dyes for 10 min, and the incubation was protected from light. The stained cells were then examined under ultraviolet light with a Hoechst filter (Nikon, Melville, NY). The percentage of live, apoptotic, and necrotic cells was recorded in eight random

fields by two observers who were unaware of the experimental conditions.

**DNA fragmentation assay: gel electrophoresis.** This is a simple method that is specific for isolation and confirmation of DNA fragments from apoptotic cells (14). Briefly, GECs grown in 100-mm petri dishes were treated as described in *Glomerular epithelial cell culture*, washed twice with PBS, and then lysed in DNA lysis buffer (1% Nonidet P-40 in 20 mM EDTA and 50 mM Tris·HCl, pH 7.5). After centrifugation, the supernatant was collected and the extraction was repeated. The supernatants were brought to 1% SDS and treated for 2 h with RNase A (final concentration of 5  $\mu$ g/ $\mu$ l) at 56°C followed by digestion with proteinase K (final concentration of 2.5  $\mu$ g/ $\mu$ l) for 2 h at 37°C. After addition of 0.5 vol of 10 M ammonium acetate, the DNA was precipitated with 2.5 vol of ethanol, dissolved in loading buffer, and separated by electrophoresis on a 1.6% agarose gel containing 10  $\mu$ g/ml ethidium bromide.

**ELISA for TGF- $\beta$ 1.** TGF- $\beta$ 1 protein from cell culture supernatants of GECs treated as described above for 6 and 18 h was measured by a TGF- $\beta$ 1-specific sandwich ELISA kit according to the manufacturer's instructions (Promega, Madison, WI). To assay for total TGF- $\beta$ 1, samples were acidified with 1 N HCl for 15 min at room temperature and then neutralized by the addition of 1 N NaOH. Results are expressed as TGF- $\beta$ 1 level in nanograms per milligram of total cell protein content.

**Protein extraction and Western blot analysis.** Protein extraction and Western blot analysis were performed as described previously (8). Briefly, after experimental treatments, GECs were washed twice with cold PBS and lysed in a modified radioimmunoprecipitation assay buffer (1  $\times$  PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM sodium orthovanadate, 0.1% SDS, 10  $\mu$ l of protease inhibitor cocktail/ml of buffer, and 100  $\mu$ g/ml phenylmethylsulfonyl fluoride) for 1 h on ice. The cell lysates were centrifuged at 15,000 g for 20 min at 4°C. The supernatant was collected, and the protein concentration of the supernatant was determined by using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL). The proteins (20  $\mu$ g/lane) were separated on a 10–15% SDS-polyacrylamide gel that was electrophoresed under reduced conditions and transferred onto a nitrocellulose membrane by using a Bio-Rad Western blot analysis apparatus. After transfer, blots and gels were stained with Ponceau S to check for complete protein transfer and equal loading. The blots were treated with 5% nonfat dried milk for 60 min at room temperature and then incubated overnight at 4°C with the primary polyclonal antibodies to Fas, FasL, Bax, Bcl-2, and AT<sub>1</sub> and AT<sub>2</sub> receptors (Santa Cruz Biotechnology, Santa Cruz, CA) followed by incubation with the corresponding horseradish peroxidase-labeled secondary antibody (Santa Cruz Biotechnology) for 1 h at room temperature. The blots were developed by using a chemiluminescence detection kit (ECL; Amersham Life Science, Arlington Heights, IL) and exposed to Kodak X-OMAT AR film. Quantitative densitometry was performed on the identified bands by using a computer-based measurement system.

**Statistical analysis.** For comparison of mean values between two individual groups, an unpaired Student's *t*-test was used. Comparison of values among multiple groups was performed by one-way ANOVA, and a Newman-Keuls multiple range analysis was used to calculate a *q*-value. Results are from four to five independent experiments, each conducted in triplicate, and are expressed as means  $\pm$  SE. Statistical significance was defined as *P* < 0.05.



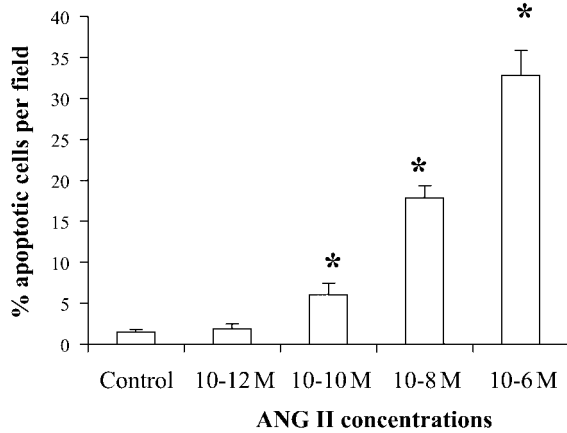


Fig. 1. Dose-response effect of ANG II on glomerular visceral epithelia cell (GEC) apoptosis. Equal numbers of subconfluent GECs were incubated in media containing either buffer (control) or variable concentrations of ANG II ( $10^{-12}$  to  $10^{-6}$  M) for 18 h. At the end of the incubation period, cells were stained with H-333342 and propidium iodide. Cells were examined in 8 random fields under a light microscope with ultraviolet light. The percentage of apoptotic cells was calculated per field. Values are means  $\pm$  SE of 4 sets of experiments, each carried out in triplicate. Effects of ANG II doses ( $10^{-10}$  to  $10^{-6}$  M) are significantly different from each other. \* $P < 0.001$  vs. control or ANG II  $10^{-12}$  M.

## RESULTS

**ANG II induces GEC apoptosis.** To evaluate the effect of ANG II on GEC apoptosis, cells were plated in 24-well plates, grown to subconfluence, and incubated with either buffer (control) or variable concentrations of ANG II ( $10^{-12}$  to  $10^{-6}$  M) for 18 h. At the end of the incubation period, cells were stained for apoptosis. As shown in Fig. 1, ANG II induced GEC apoptosis in a dose-dependent manner. At a higher concentration ( $10^{-6}$  M), ANG II also induced GEC necrosis ( $5.6 \pm 1.6$  vs.  $0.3 \pm 0.3\%$  of control,  $P < 0.05$ ). We then evaluated the time course during which ANG II could induce GEC apoptosis. As shown in Fig. 2, ANG II ( $10^{-8}$  M) promoted GEC apoptosis in a time-dependent manner.

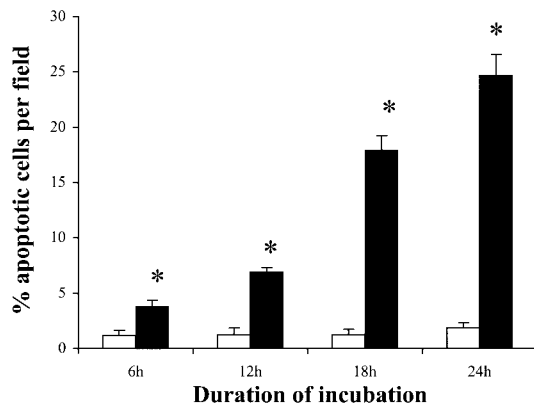


Fig. 2. Time course effect of ANG II on GEC apoptosis. Subconfluent GECs were incubated in media containing either buffer (control) or ANG II ( $10^{-8}$  M) for variable time periods (6, 12, 18, and 24 h) and then stained for apoptosis. Values are means  $\pm$  SE of 4 sets of experiments. All ANG II-treated time points are significantly different from each other. \* $P < 0.001$  vs. respective control.

Figure 3 shows a representative morphological view of ANG II-induced GEC apoptosis.

GECs were also grown to confluence and treated with ANG II ( $10^{-8}$  M) to evaluate the effect of cell density on ANG II-induced apoptosis. There was no significant difference in ANG II-induced apoptosis between cells grown to confluence and subconfluent cells (data not shown).

To exclude the possibility that ANG II was hydrolyzed into fragments (4) that led to the cell apoptosis, GECs were incubated with ANG III, ANG IV, or ANG-(1-7) ( $10^{-8}$  M) for 18 h. None of these fragments significantly induced apoptosis compared with control [control,  $1.0 \pm 0.4$ ; ANG III,  $1.6 \pm 0.5$ ; ANG IV,  $4.0 \pm 1.5$ ; ANG-(1-7),  $1.2 \pm 0.5\%$  apoptotic cells/field,  $n = 4$ ].

By Western blot analysis, we have confirmed the presence of AT<sub>1</sub> and AT<sub>2</sub> receptor expression in GECs. These are detected as protein bands of  $\sim 41/53$  and  $\sim 44$  kDa, respectively.

**Effects of ANG II receptor antagonists on ANG II-induced apoptosis.** To determine whether AT<sub>1</sub> or AT<sub>2</sub> receptors are involved in the mediation of ANG II-induced apoptosis, GECs were pretreated for 30 min with either losartan ( $10^{-6}$  M), a selective AT<sub>1</sub> receptor antagonist, or PD-123319 ( $10^{-6}$  M), a selective AT<sub>2</sub>

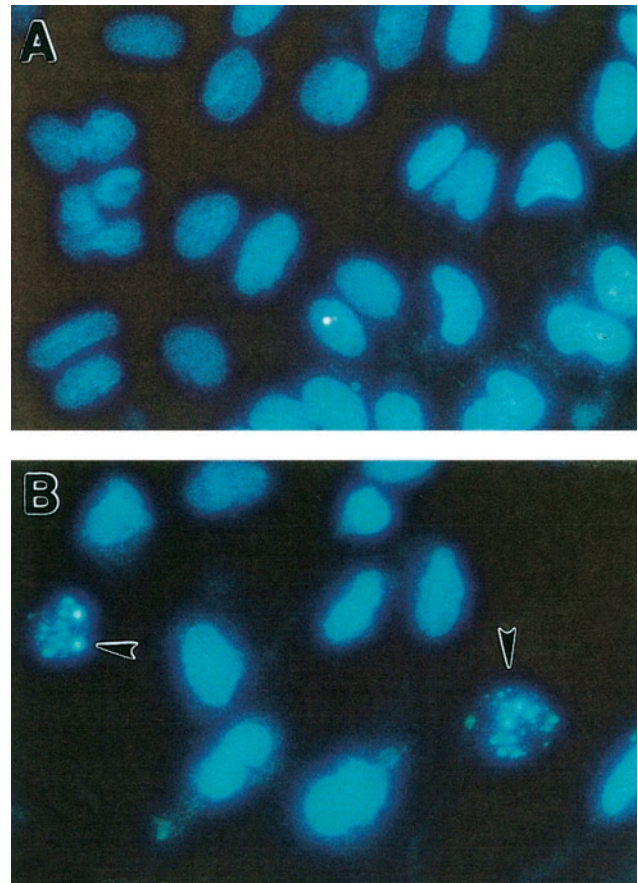


Fig. 3. Effect of ANG II on GEC apoptosis. A: control GECs show nuclei with moderate fluorescence. B: GECs treated with ANG II ( $10^{-8}$  M) show nuclear condensation and fragmentation. Arrowheads, apoptosis. Magnification,  $\times 150$ .

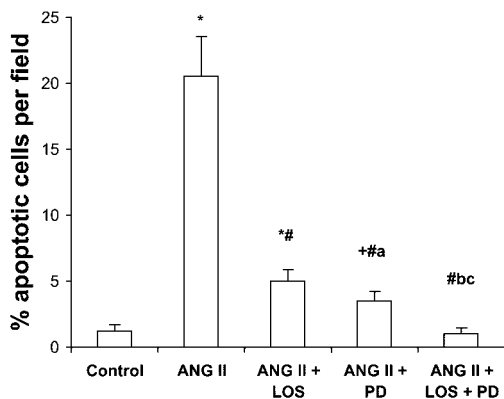


Fig. 4. Effects of ANG II receptor antagonists on ANG II-induced cell apoptosis. GECs were incubated in media containing either buffer (control) or ANG II ( $10^{-8}$  M) in the absence or presence of losartan (LOS) or PD-123319 (PD) or losartan + PD-123319. Cells were then stained for apoptosis. Results (means  $\pm$  SE) are from 5 sets of experiments. \* $P < 0.001$  vs. control; + $P < 0.05$  vs. control; # $P < 0.001$  vs. ANG II treatment alone; #a $P < 0.05$  vs. ANG II + losartan; #b $P < 0.001$  vs. ANG II + losartan; #c $P < 0.05$  vs. ANG II+PD-123319.

receptor antagonist, or with a combination of both and then treated with the addition of ANG II ( $10^{-8}$  M) for 18 h. As shown in Fig. 4, addition of losartan or PD-123319 significantly inhibited ANG II-induced GEC apoptosis, but the addition of both receptor antagonists prevented it.

**Effect of ANG II on TGF- $\beta$ 1 protein production in GECs.** To study the effect of ANG II on TGF- $\beta$ 1 protein production, GECs were incubated with ANG II ( $10^{-8}$  M) in the presence or absence of losartan ( $10^{-6}$  M) or PD-123319 ( $10^{-6}$  M), and measurement of TGF- $\beta$ 1 protein in cultured supernatants was performed by using a specific ELISA. As shown in Fig. 5, ANG II stimulated TGF- $\beta$ 1 secretion by GECs at 6 and 18 h (2.0- and 1.7-fold increases vs. respective control,  $P < 0.05$ ). ANG II-stimulated TGF- $\beta$ 1 production was sig-

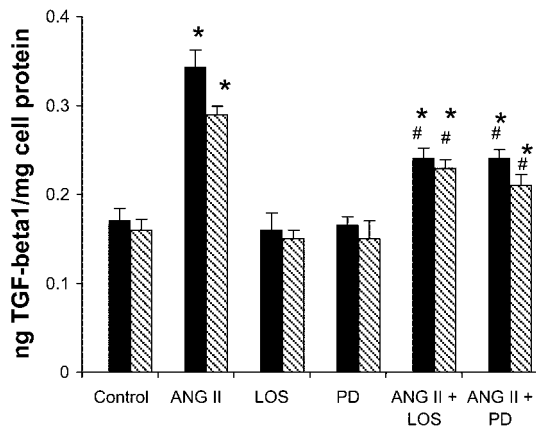


Fig. 5. Effect of ANG II on transforming growth factor (TGF)- $\beta$ 1 protein production in the presence or absence of losartan or PD-123319. After incubation of GECs under various experimental conditions for 6 (filled bars) and 18 h (hatched bars), TGF- $\beta$ 1 protein secretion in the cell culture supernatants was determined by ELISA. Values are means  $\pm$  SE of 4 separate experiments. \* $P < 0.05$  vs. respective control; # $P < 0.05$  vs. respective ANG II treatment alone.

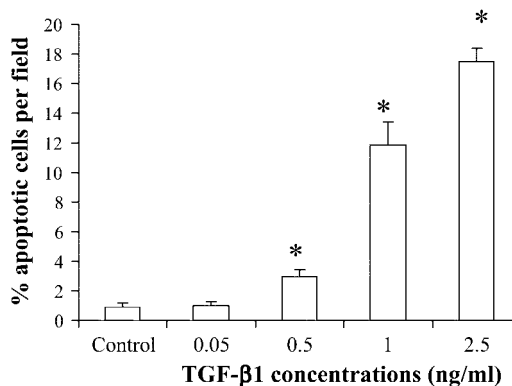


Fig. 6. Dose-response effect of TGF- $\beta$ 1 on GEC apoptosis. GECs were incubated in media containing either buffer (control) or variable doses of TGF- $\beta$ 1 (0.05, 0.5, 1, and 2.5 ng/ml) for 18 h and then stained for apoptosis. Values are means  $\pm$  SE of 4 sets of experiments. Effects of TGF- $\beta$ 1 doses (0.5 to 2.5 ng/ml) are significantly different from each other. \* $P < 0.001$  vs. control or TGF- $\beta$ 1 (0.05 ng/ml).

nificantly diminished in the presence of losartan or PD-123319.

**Effect of anti-TGF- $\beta$ 1 antibody on ANG II-induced GEC apoptosis.** We first evaluated the effect of TGF- $\beta$ 1 on GEC apoptosis. As shown in Fig. 6, TGF- $\beta$ 1 in concentrations  $\geq 0.5$  ng/ml significantly enhanced GEC apoptosis (TGF- $\beta$ 1 0.5 ng/ml,  $2.9 \pm 0.4\%$ ; 1 ng/ml,  $11.9 \pm 1.5\%$ ; and 2.5 ng/ml,  $17.5 \pm 0.9\%$  apoptotic cells/field) compared with control ( $0.9 \pm 0.3\%$  apoptotic cells/field,  $P < 0.05$ ,  $n = 4$ ). TGF- $\beta$ 1 also enhanced GEC apoptosis in a time-dependent manner (Fig. 7).

To investigate whether the newly produced TGF- $\beta$ 1 was involved in the mediation of ANG II-induced cell apoptosis, GECs were incubated with ANG II ( $10^{-8}$  M) in the presence of anti-TGF- $\beta$ 1 antibody (5  $\mu$ g/ml) for 18 h. As shown in Fig. 8, addition of anti-TGF- $\beta$ 1 antibody significantly attenuated the ANG II-induced GEC apoptosis [ANG II  $10^{-8}$  M,  $18.2 \pm 1.4$ ; ANG II  $10^{-8}$  M+anti-TGF- $\beta$ 1 antibody (5  $\mu$ g/ml),  $5.4 \pm 1.0\%$  apoptotic cells/field,  $P < 0.05$ ,  $n = 4$ ].

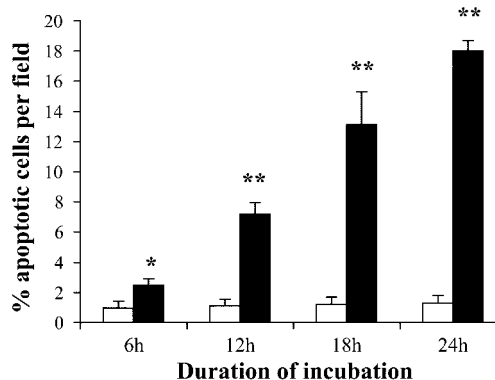


Fig. 7. Time course effect of TGF- $\beta$ 1 on GEC apoptosis. GECs were incubated in media containing either buffer (control) or TGF- $\beta$ 1 (1 ng/ml) for variable time periods (6, 12, 18, and 24 h) and then stained for apoptosis. Values are means  $\pm$  SE of 4 sets of experiments. \* $P < 0.05$  vs. control at 6 h; \*\* $P < 0.001$  vs. control at 12, 18, and 24 h.

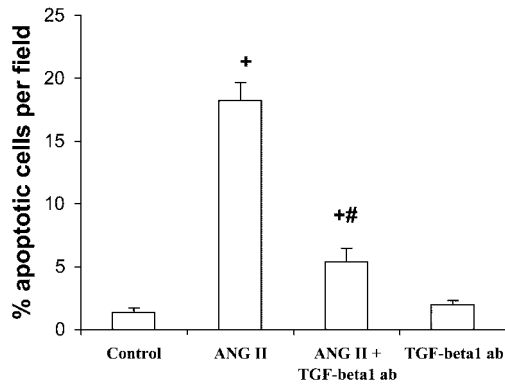


Fig. 8. Effect of anti-TGF- $\beta$ 1 antibody (ab) on ANG II-induced GEC apoptosis. Cells were incubated in media containing either buffer (control) or ANG II ( $10^{-8}$  M) in the absence or presence of anti-TGF- $\beta$ 1 antibody or with the antibody alone. Values are means  $\pm$  SE of 4 sets of experiments. + $P$  < 0.001 vs. control; # $P$  < 0.001 vs. ANG II alone.

**DNA fragmentation assay.** DNA gel electrophoresis was used to confirm the formation of low-molecular-weight DNA fragments in the apoptotic GECs. As illustrated in Fig. 9, ANG II-treated cells showed increased integer multiples of 180 bp in the form of a ladder pattern. The effect of ANG II on DNA fragmentation in GECs was partially prevented by the addition of the AT<sub>1</sub> receptor antagonist losartan or the AT<sub>2</sub> receptor antagonist PD-123319 and completely prevented by the addition of both receptor antagonists. TGF- $\beta$ 1 antibody also attenuated ANG II-induced DNA fragmentation in GECs.

**Effect of ANG II on Fas, FasL, Bax, and Bcl-2 production.** Altered expression of cell death and cell survival proteins such as Fas, FasL, Bax, and Bcl-2 has been demonstrated to play an important role in the induction of apoptosis; therefore, we evaluated the effects of ANG II and its receptor antagonists on expression in GECs of these apoptosis-related proteins by Western blot analysis. As illustrated, ANG II significantly enhanced Fas (Fig. 10A), FasL (Fig. 10B), and Bax (Fig. 10C) in GECs. On the contrary, Bcl-2 expression was downregulated in response to ANG II stimulation (Fig. 10D). However, in the presence of either losartan or PD-123319, the increments in Fas, FasL, and Bax expression were inhibited. In contrast, ANG II-induced downregulation of the Bcl-2 level was inhibited only by losartan and not by PD-123319.

## DISCUSSION

The present study shows that ANG II induces GEC apoptosis in a dose- and time-dependent manner. ANG II also induced necrosis in GEC at the higher concentration. ANG II-induced apoptosis was prevented by simultaneous incubation of both losartan and PD-123319. Furthermore, ANG II stimulated TGF- $\beta$ 1 production and anti-TGF- $\beta$ 1 antibody attenuated the ANG II-induced GEC apoptosis. To our knowledge, the present investigation is the first demonstration that apoptosis occurs in cultured cells of glomerular origin in response to ANG II.

Numerous studies have suggested that ANG II plays an important role in the development of glomerulosclerosis (23, 43, 47). The mechanisms by which ANG II contributes to the pathological process have been attributed to its hemodynamic and nonhemodynamic effects (or direct effects) (2, 10, 42). As Lapinski et al. (21) reported in the isolated perfused kidney, continuous infusion of ANG II induced a loss of glomerular size permselectivity and an increase in urinary protein excretion rate. Arai et al. (3) demonstrated glomerular sclerosis in rats by transfection of genes for renin and angiotensinogen into the kidney. In vivo administration of ANG II to animals significantly increased the accumulation of ECM proteins and expression of TGF- $\beta$  in the glomeruli (15). In vitro ANG II treatment promoted renal cell growth associated with an increase in the synthesis of ECM proteins (10, 43).

Glomerulosclerosis is characterized by progressive accumulation of ECM and loss of resident glomerular cells (11, 19, 28). Recent data suggest that an imbalance between cell survival and death may result in a loss of glomerular cells, which underlies the development of glomerulosclerosis (35, 39, 46, 48). ANG II, in addition to its growth-promoting effects, has been demonstrated to induce apoptosis in several nonrenal cells in culture (7, 16, 42, 44, 45). In the present study, we tested whether ANG II affects the survival of GECs in culture. Our data clearly indicate that ANG II induces GEC apoptosis in a dose- and time-dependent manner. Because GECs have been shown to express both AT<sub>1</sub> and AT<sub>2</sub> receptors (36), we then determined whether AT<sub>1</sub> or AT<sub>2</sub> receptors are involved in the mediation of the ANG II-induced apoptosis. To our surprise, the proapoptotic effects of ANG II were only partially blocked by treatment with either losartan or PD-123319 but were completely blocked by their combination, suggesting that ANG II-induced GEC apoptosis is associated with activation of both receptors. It is generally considered that the proapoptotic effects of ANG II are mediated by the AT<sub>2</sub> receptor, whereas

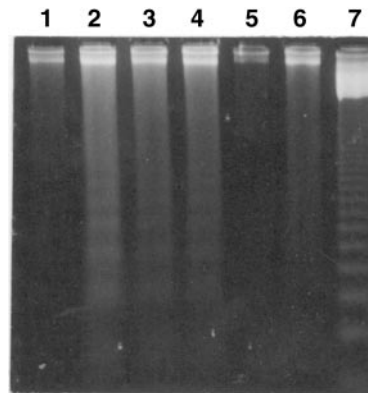
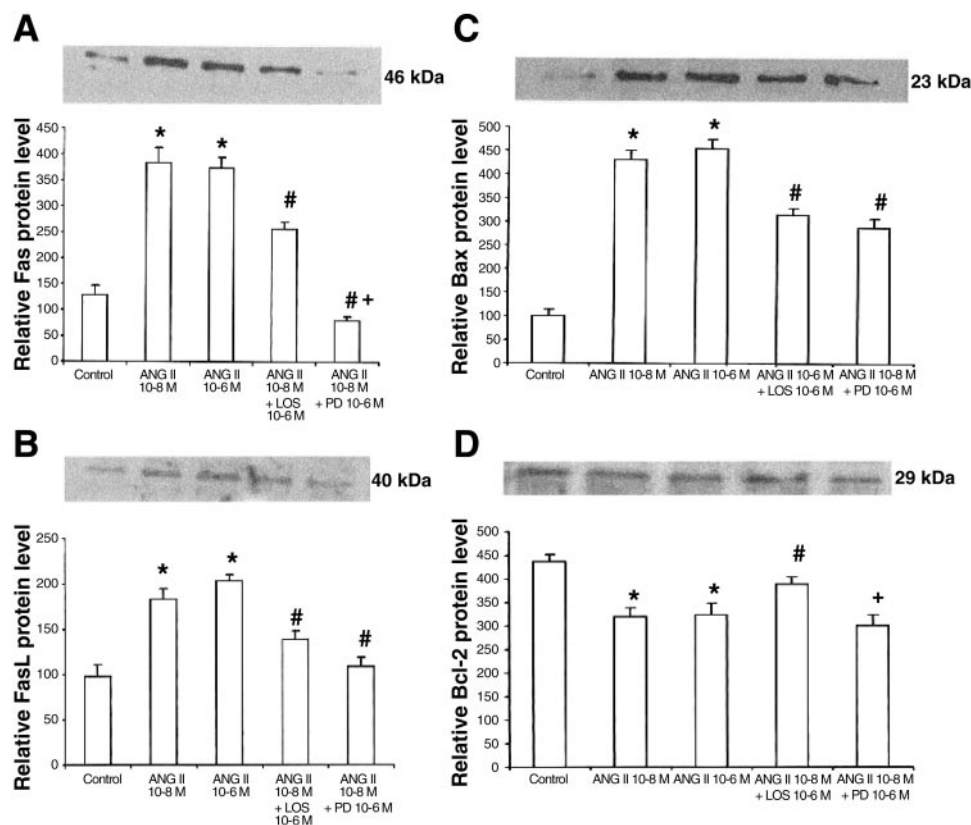


Fig. 9. Effects of ANG II receptor antagonists and anti-TGF- $\beta$ 1 antibody on ANG II-induced DNA fragmentation. Lane 1: control; lane 2: multiple integers of 180 bp in ANG II-treated GECs; lanes 3 (ANG II + LOS), 4 (ANG II + PD), and 5 (ANG II + LOS + PD): effect of receptor antagonists on ANG II-induced DNA fragmentation; lane 6: GECs treated with ANG II in the presence of anti-TGF- $\beta$ 1 antibody; lane 7: molecular marker.



Fig. 10. Effects of ANG II with and without losartan or PD-123319 on Fas (A), FasL (B), Bax (C), and Bcl-2 (D). ANG II ( $10^{-8}$  M, 18 h) upregulates the production of Fas, FasL, and Bax but downregulates the production of Bcl-2. Cotreatment with losartan or PD-123319 inhibits ANG II-stimulated Fas, FasL, and Bax. ANG II-induced reduction of Bcl-2 is only inhibited by losartan. \* $P < 0.05$  vs. control; # $P < 0.05$  vs. ANG II  $10^{-8}$  M; + $P < 0.05$  vs. ANG II + losartan.



the growth-promoting effects are mediated by the  $AT_1$  receptor (4). However, recent studies of myocytes demonstrated the involvement of the  $AT_1$  receptor in apoptosis (22). The results of the present investigation indicate that both  $AT_2$  and  $AT_1$  receptors are involved in ANG II-induced GEC apoptosis. Our findings are consistent with recent data showing that both receptors have similar effects on apoptosis in ANG II-treated human endothelial cells (7) and in ANG II-infused rat kidneys (6). It is possible that ANG II may induce cross-talk between the  $AT_1$  and  $AT_2$  receptors or stimulate a common event of the two receptor pathways, such as the production of a given mediator or cytokine, that leads to cell apoptosis (6, 7).

GECs, or podocytes, are one of the major cell types within the glomerulus and play an important role in maintaining normal structure and function of the glomerular basement membrane. Under pathological conditions, GECs would be subjected to the detrimental effects of intrarenal ANG II activation. Injury or loss in GECs may be the sequelae of ANG II-induced apoptosis. Our present studies extend the role of ANG II as a proapoptotic cytokine to contribute to the pathobiology of glomerulosclerosis.

It has been shown that GECs cultured from human as well as rat kidneys produce TGF- $\beta_1$  (9, 41). The results of the present study demonstrate that ANG II stimulates TGF- $\beta_1$  protein synthesis in rat GECs. Moreover, both  $AT_1$  and  $AT_2$  receptor antagonists significantly inhibit ANG II-induced TGF- $\beta_1$  production. Thus the signal transduction of the ANG II effect is

also considered to occur through both  $AT_1$  and  $AT_2$  receptors. The coincidence of ANG II-stimulated TGF- $\beta_1$  expression and cell apoptosis through the signal transduction of both receptor types suggests that TGF- $\beta_1$  may be an important mediator of ANG II-induced GEC apoptosis.

TGF- $\beta$  is a multifunctional growth factor that can either inhibit or stimulate cell proliferation and growth. We have recently reported that TGF- $\beta$  promotes apoptosis in glomerular mesangial cells in vitro (27). TGF- $\beta$  is also involved in apoptosis of renal tubular cells exposed to a mechanical stretch (24) and in apoptosis of podocytes in transgenic mice (32). In the present study, we found that TGF- $\beta_1$  treatment was associated with a dose- and time-dependent enhancement of GEC apoptosis. We further hypothesized that the newly produced TGF- $\beta$ , in response to ANG II, may be involved in cell apoptosis. When GECs were exposed to ANG II in the presence of neutralizing antibody to TGF- $\beta_1$ , we noticed an obvious reduction in apoptosis. These results support our hypotheses that ANG II stimulates TGF- $\beta_1$  production and that the newly synthesized TGF- $\beta_1$  is involved in cell apoptosis.

Both Fas and FasL have been implicated in the induction of increased cell apoptosis in glomerular injury (26). In the present study, we have shown that ANG II-induced apoptosis is associated with enhanced expression of Fas and FasL in GECs. In addition, ANG II induced an accumulation of Bax, an apoptosis-promoting factor (25), and a reduction of Bcl-2, an apoptosis-preventing factor (1). We have further dem-

onstrated that ANG II-stimulated protein levels of Fas, FasL, and Bax are inhibited by treatment with either losartan or PD-123319, raising the possibility that both AT<sub>1</sub> and AT<sub>2</sub> receptor transduction pathways are involved in the induction of Fas, FasL, and Bax. In contrast, ANG II-induced reduction of Bcl-2 is specific only for the AT<sub>1</sub> receptor, because losartan, not PD-123319, modulates the ANG II effect on Bcl-2 expression. However, our data on altered expression of the apoptosis-related proteins specific for ANG II receptor transduction may provide the molecular basis for the enhanced cell susceptibility to apoptosis.

Recently, Saleh et al. (30) reported that thapsigargin triggered mesangial cell apoptosis through the induction of a sustained increase of cytoplasmic-free concentration of calcium. On the other hand, platelet-derived growth factor, which is known to elevate mesangial cell cytosolic calcium, inhibited thapsigargin-induced mesangial cell apoptosis. Saleh et al. suggested that the time and duration of the cytosolic calcium peak may determine whether a cell will enter into a proliferative or apoptotic phase. At present, data on cytosolic calcium in glomerular epithelial cells in response to various vasoactive agents are scanty and worth pursuing in future studies.

In summary, we have shown that ANG II can induce GEC apoptosis in culture and that the proapoptotic effects are mediated by both AT<sub>1</sub> and AT<sub>2</sub> receptors. Our studies further demonstrate the involvement of TGF- $\beta$ 1 and an altered expression of apoptotic regulatory proteins in the ANG II-induced apoptosis. These findings may indicate an important role for ANG II as a proapoptotic cytokine contributing to the pathobiology of glomerulosclerosis.

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